

# The Role of Shared Receptor Motifs and Common Stat Proteins in the Generation of Cytokine Pleiotropy and Redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15

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et al., 1993a; Kondo et al., 1994), IL-9 (Russell et al., 1994), and IL-15 (Giri et al., 1994) receptors. IL-2, IL-4, IL-7, IL-9, and IL-15 can all act as T cell growth factors, thereby exhibiting cytokine redundancy, the ability of multiple cytokines to induce similar actions (Paul, 1989; Leonard, 1994). IL-2, IL-4, IL-7, and IL-9 have been shown to activate two Janus family tyrosine kinases, Jak1 and Jak3 (Johnston et al., 1994; Witthuhn et al., 1994; Russell et al., 1994). Jak kinases play critical signaling roles for interferon (IFN) $\alpha/\beta$ , IFN $\gamma$ , and for a number of members of the cytokine receptor superfamily by activating signal transducers and activators of transcription (Stat) proteins (Ihle et al., 1994; Darnell et al., 1994). To elucidate the molecular bases of cytokine pleiotropy and redundancy, we analyzed the Stat proteins activated by a number of cytokines with shared and distinct biological activities.

## Summary

To understand the molecular bases for cytokine redundancy and pleiotropy, we have compared the Stat proteins activated in peripheral blood lymphocytes (PBLs) by cytokines with shared and distinct actions. Interleukin-2 (IL-2) rapidly activated Stat5 in fresh PBL, and Stat3 and Stat5 in preactivated PBL. IL-7 and IL-15 induced the same complexes as IL-2, a feature explained by the existence of similar tyrosine-phosphorylated motifs in the cytoplasmic domains of IL-2R $\beta$  and IL-7R that can serve as docking sites for Stat proteins. IL-13 induced the same complexes as IL-4, a finding explained by our studies implicating IL-4R as a shared component of the receptors. These studies demonstrate that a single cytokine can activate different combinations of Stat proteins under different physiological conditions, and also indicate two mechanisms by which distinct cytokines can activate the same Stat protein.

## Introduction

The term "cytokine pleiotropy" describes the ability of a cytokine to exert more than one action, often on multiple cell types (Paul, 1989; Leonard, 1994). For example, IL-2 can induce or augment the cytolytic activity of natural killer (NK) cells, the proliferation of T cells, and immunoglobulin biosynthesis by B cells (Leonard et al., 1994; Taniguchi and Minami, 1993; Waldmann, 1989). Functional IL-2 receptors contain IL-2R $\beta$  and the common  $\gamma$  chain ( $\gamma_c$ ) (reviewed by Leonard, 1994; Leonard et al., 1994; Taniguchi and Minami, 1993), two cytokine receptor superfamily members (Bazan, 1990) whose dimerization is required for signaling (Nakamura et al., 1994; Nelson et al., 1994).

Mutations of the  $\gamma_c$  gene can result in X-linked severe combined immunodeficiency (XSCID) in humans (Noguchi et al., 1993b); the severity of this disease results from  $\gamma_c$  being a component of the IL-2 (Takeshita et al., 1992), IL-4 (Kondo et al., 1993; Russell et al., 1993), IL-7 (Noguchi

## Results

### Activation of Stat Proteins by IL-2 in Fresh and Preactivated PBL and in CTLL-2 Cells

We first evaluated the activation of nuclear factors by IL-2 using electrophoretic mobility shift assays (EMSAs) and a probe corresponding to an IFN $\gamma$ -activated site (GAS) from the Fc $\gamma$ RI gene (core site, 5'-GTATTTCCCAAGG-3'). IL-2 rapidly induced nuclear binding activity in freshly isolated peripheral blood lymphocytes (PBLs) (Figure 1A, lanes 1–4). When PBLs were preactivated by phytohemagglutinin (PHA) for 72 hr and then rested overnight, little or no binding activity was detected; however, IL-2 rapidly induced two complexes (Figure 1A, lanes 5–8), the upper component of which had similar mobility to the complex induced by IL-2 in YT cells (lanes 9–12). The complexes induced were specific in that they could be competed by cold competitor (data not shown) and were not formed with an oligonucleotide containing a single nucleotide change (TTCCCACAA instead of TTCCCAAGG) in the core of the GAS motif (Figure 1A, lanes 13–16), indicating that the GAS motif is essential for binding and therefore suggesting that Stat proteins were involved in the formation of the complexes. As expected for Stat proteins, DNA binding activity was diminished when extracts from fresh PBL, preactivated PBL, or YT cells were treated with protein tyrosine phosphatase (Figure 1B). Furthermore, for both fresh and preactivated PBL, the binding activities were induced rapidly in the cytosol, and cycloheximide did not inhibit the activation of these binding activities (data not shown), consistent with the activation of preformed cytosolic Stat proteins (Darnell et al., 1994; Ihle et al., 1994). IL-2 also activated Stat proteins in CTLL-2 cells (Figure 1C, lanes 1–4), correlating with their vigorous IL-2-induced responsiveness. Although rapamycin inhibits certain IL-2 signals (Kuo et al., 1992; Morice et al., 1993), it did not affect either the activation or nuclear translocation of IL-2-activated Stat proteins (data not shown).

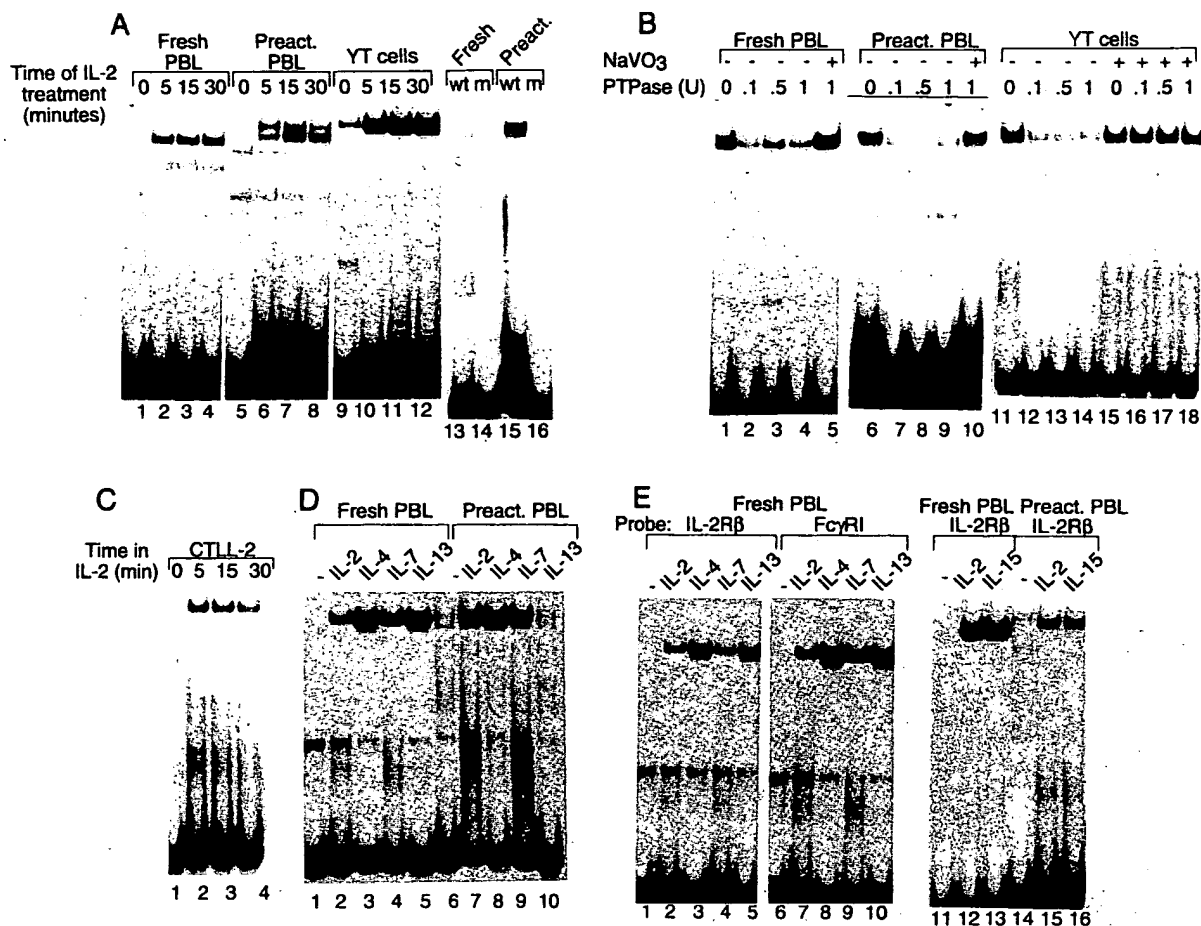


Figure 1. Rapid Induction of Nuclear Binding Activities by IL-2, IL-4, IL-7, IL-13, and IL-15

(A) IL-2 rapidly induced nuclear complexes in freshly isolated PBL (lanes 1–4), preactivated PBL (lanes 5–8), and YT cells (lanes 9–12). No complexes were detected when the GAS motif was mutated (lanes 14 and 16 versus lanes 13 and 15). (B) Treatment of extracts from fresh PBL (lanes 1–5), preactivated PBL (lanes 6–10), or YT cells (lanes 11–18) with 0.1, 0.5, or 1.0 U of T cell phosphatase (recombinant human T cell protein tyrosine phosphatase; New England Biolabs) for 30 min at 30°C diminished the IL-2-activated binding activity (lanes 2–4, 7–9, and 12–14) unless inhibited by sodium vanadate (lanes 5, 10, 16–18). (C) Stat proteins were activated when CTLL-2 were stimulated with IL-2 for 5–30 min (lanes 1–4). (D) Induction of nuclear binding activity by IL-2, IL-4, IL-7, and IL-13 in fresh PBL (lanes 1–5) and by IL-2, IL-4, and IL-7, but not IL-13 in preactivated PBL (lanes 6–10). The FcγRI probe (5'-AGCTTGTATTTCAGAAAGGGATC-3', GAS motif underlined) was used in (A)–(D). (E) EMSAs with the IL-2Rβ (5'-AGCTTGAATTCATGGAATGGGATC-3', GAS motif underlined) (lanes 1–5) and FcγRI probe (lanes 6–10) using IL-2, IL-4, IL-7, or IL-13 activated nuclear extracts derived from fresh PBL. IL-15-induced complexes were compared with IL-2-induced complexes in fresh (lanes 11–13) and preactivated (lanes 14–16) PBL.

#### Comparison of IL-2-, IL-4-, IL-7-, IL-13-, and IL-15-Induced Complexes

We next compared the Stat proteins activated by IL-2 to those activated by IL-4 and IL-7, two other T cell growth factors known to share *γ<sub>c</sub>* and activate Jak1 and Jak3. Whereas IL-2 and IL-7 induced complexes of identical mobility, IL-4 induced a complex with a different appearance (Figure 1D). The protein(s) forming the complex(es) induced by IL-4 have been denoted as IL-4 NAF (Kotianides and Reich, 1993), STF-IL-4 (Schindler et al., 1994), and IL-4 Stat (Hou et al., 1994). IL-13, which shares many actions with IL-4 (Zurawski et al., 1993; Zurawski and de Vries, 1994), induced a complex identical in mobility to that induced by IL-4, but only in fresh PBL (Figure 1D), consistent with the inability of IL-13 to stimulate activated T cells (Zurawski and de Vries, 1994). The IL-2-, IL-4-,

IL-7-, and IL-13-induced complexes were also formed with a probe corresponding to a GAS motif found in the IL-2Rβ promoter (Gnarra et al., 1990) (Figure 1E, lanes 1–10), a gene induced by both IL-2 (Siegel et al., 1987) and IL-4 (Casey et al., 1992). This probe was additionally used to demonstrate that IL-15 induced complexes indistinguishable from those induced by IL-2 in fresh and preactivated PBL (Figure 1E, lanes 11–16). Interestingly, the IL-2Rβ motif (TTCATGGAA, -854 to -846 in the coordinate system of Lin et al., 1993; the inverted repeat is underlined) was more potent than the FcγRI motif (TTCAGAA) in its ability to activate transcription of the TK promoter in response to IL-2 in transiently transfected YT cells (data not shown). In preactivated PBL stimulated with IL-2, the IL-2Rβ probe always yielded a single complex (Figure 1E), in contrast with the doublet seen with the FcγRI probe

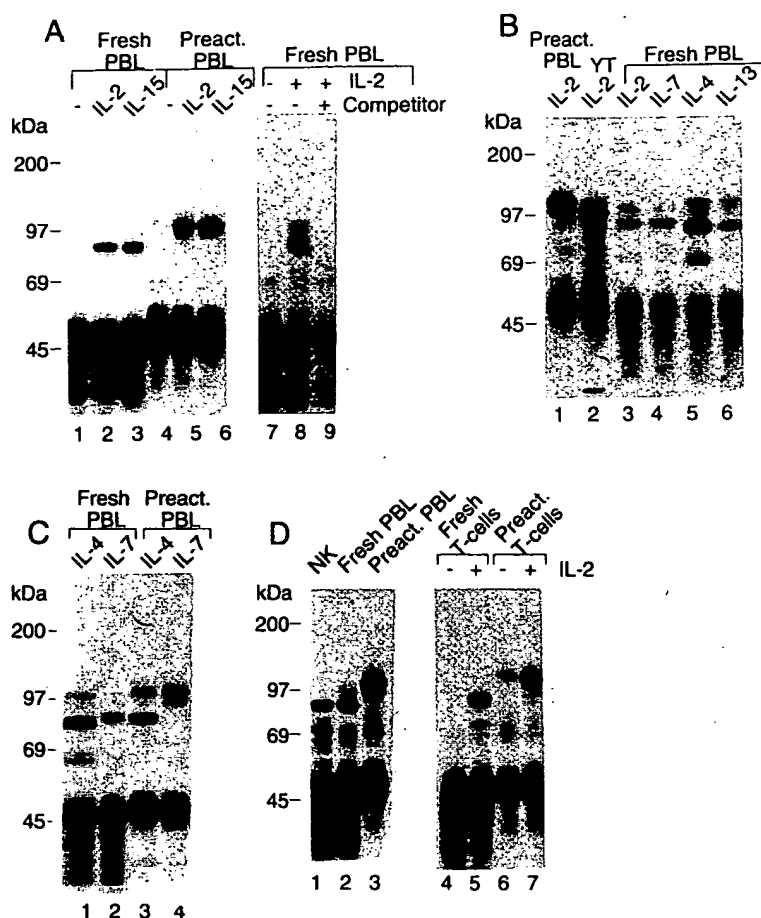


Figure 2. UV Cross-Linking Reveals Identity of IL-2-, IL-7-, and IL-15-Activated Factors and of IL-4- and IL-13-Activated Factors

(A) Freshly isolated PBLs were not treated (lanes 1, 7), or treated with IL-2 (lanes 2, 8, 9), or IL-15 (lane 3), extracts were prepared and UV cross-linking was performed. In lane 9, 25 ng of unlabeled probe was added prior to UV cross-linking. Preactivated PBLs were not treated (lane 4) or treated with IL-2 (lane 5) or IL-15 (lane 6) prior to UV cross-linking.

(B) Preactivated PBL (lane 1) and YT cells (lane 2) were stimulated with IL-2. Freshly isolated PBL were stimulated with IL-2 (lane 3), IL-7 (lane 4), IL-4 (lane 5), or IL-13 (lane 6), and extracts subjected to UV cross-linking using the IL-2R $\beta$  probe. In IL-2-stimulated fresh PBL, a primary band of 87.5 kDa and a fainter 96/102.5 kDa doublet were detected; in IL-4-stimulated fresh PBL the major band migrated at 85 kDa, with minor bands of 65 and 102 kDa.

(C) Comparison of UV cross-linked adducts generated using extracts from fresh (lanes 1 and 2) and preactivated PBL (lanes 3 and 4) stimulated with IL-4 (lanes 1 and 3) and IL-7 (lanes 2 and 4).

(D) UV cross-linked adducts formed with nuclear extracts from IL-2-stimulated purified NK cells (lane 1), and IL-2-stimulated fresh (lane 2) or preactivated (lane 3) PBL. UV cross-linked adducts formed with nuclear extracts from fresh purified T cells not treated (lane 4) or treated (lane 5) with IL-2, and preactivated purified T cells not treated (lane 6) or treated (lane 7) with IL-2.

(Figure 1A); nevertheless, similar ultraviolet (UV) cross-linked adducts were seen with the IL-2R $\beta$  (see below) and Fc $\gamma$ RI (data not shown) probes. Thus, fine sequence variations within the core and flanking sequences of GAS-like sites are critical to cytokine-specific responsiveness.

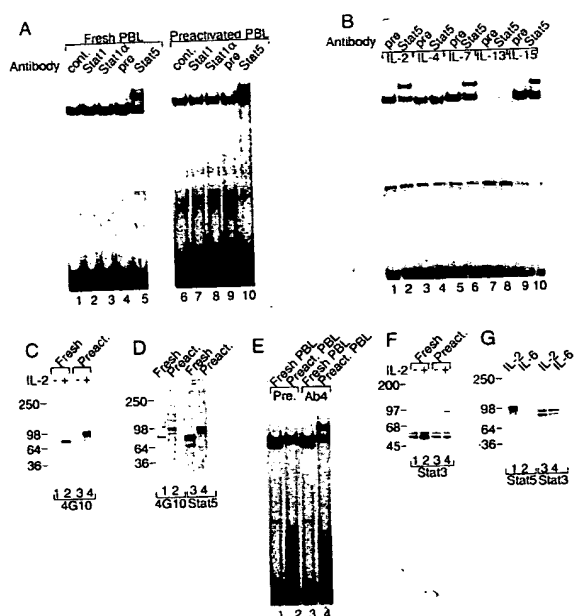
#### UV Cross-Linking of IL-2-, IL-4-, IL-7-, IL-13-, and IL-15-Induced Complexes

To analyze further the complexes induced by IL-2, IL-4, IL-7, IL-13, and IL-15, we used a bromodeoxyuridine-containing  $^{32}$ P-labeled IL-2R $\beta$  probe and UV cross-linking methodology. As expected, identical patterns were seen with extracts from fresh PBL stimulated with IL-2, IL-7, or IL-15 (Figure 2A, 1-3; Figure 2B, lanes 3 and 4); these UV cross-linked adducts were specific based on their absence in uninduced cells and by their competition by excess unlabeled probe (Figure 2A, lanes 7-9). In contrast, cells stimulated with IL-4 or IL-13 yielded a different pattern (Figure 2B, lanes 5 and 6). For IL-2, IL-7, and IL-15, it was striking that the migration of the UV cross-linked adducts in preactivated cells differed dramatically from the major adduct seen in fresh PBL (Figure 2A, lanes 5 and 6 versus 2 and 3; Figure 2C, lane 4 versus lane 2). The differences between the UV cross-linked adducts in fresh and preactivated PBL stimulated with IL-4 were much more subtle (Figure 2C, lane 3 versus lane 1). Since IL-2 can induce

signals in freshly isolated NK cells and T cells (reviewed by Leonard and Siegel, 1990), we hypothesized that both cell types might contribute to the binding activity induced by IL-2 in fresh PBL. Indeed, the major UV cross-linked adducts seen in freshly isolated NK and T cells stimulated with IL-2 were the same as those seen in fresh PBL stimulated with IL-2 (Figure 2D, lanes 1, 2, 5), and IL-2-stimulated preactivated T cells gave the same pattern as did preactivated PBL (Figure 2D, lanes 3 and 7).

#### Activation by IL-2 of Stat5 in Fresh PBL and Both Stat3 and Stat5 in Preactivated PBL

We next investigated the identities of the Stat proteins in the complex induced by IL-2. Antisera reactive with Stat1 (both Stat1 $\alpha$  and Stat1 $\beta$ ) did not react with the IL-2-induced complexes (Figure 3A, lanes 2-3, 7-8), even though they could block formation of the complex induced by IFN $\alpha$  (data not shown). However, an antiserum to Stat5 supershifted the complexes induced by IL-2 in both fresh and preactivated PBL (Figure 3A, lanes 5 and 10), indicating that these complexes contained Stat5. Ovine Stat5 was first isolated as a Stat protein activated by prolactin (Wakao et al., 1994). As expected, the Stat5 antiserum also supershifted the complexes induced by IL-7 and IL-15, but had no effect on those induced by IL-4 and IL-13 (Figure



**Figure 3.** Activation of Stat5 by IL-2, IL-7, and IL-15 in Freshly Isolated and Preactivated PBL, and Stat3 by IL-2 Only in Preactivated PBL. (A) R1146 anti-Stat5 antiserum supershifted the IL-2-induced complexes in both fresh and preactivated PBL (lanes 5 and 10), whereas antibodies to Stat1 and Stat1 $\alpha$  had no effect. The control preimmune serum (pre) is shown for the Stat5 antiserum. (B) R1146 antiserum to Stat5 supershifted the complexes induced by IL-2, IL-7, and IL-15, but not those induced by IL-4 and IL-13 in fresh PBL. (C) Anti-phosphotyrosine Western blotting of DNA affinity-purified complexes from fresh (lanes 1 and 2) and preactivated (lanes 3 and 4) PBL not stimulated (lanes 1 and 3) or stimulated (lanes 2 and 4) with IL-2. In fresh PBL, a major band of 80 kDa and minor bands of 90 and 60 kDa were identified. The 60 kDa band in lane 2 was variably detected and could represent a degradation product. For preactivated PBL, major and minor bands of 95 and 90 kDa were identified. (D) Anti-phosphotyrosine (lanes 1 and 2) or anti-Stat5 (lanes 3 and 4) Western blotting of DNA affinity-purified complexes from fresh (lanes 1 and 3) and preactivated (lanes 2 and 4) PBL stimulated with IL-2. (E) Ab4 (raised to amino acids 551–607 of Stat1), supershifted the IL-2-induced complex in preactivated (lane 4) but not in fresh (lane 3) PBL. Preimmune serum (pre) had no effect (lanes 1 and 2). (F) Lysates from fresh (lanes 1 and 2) and preactivated (lanes 3 and 4) PBL not stimulated (lane 1 and 3) or stimulated (lanes 2 and 4) with IL-2 were immunoprecipitated with 4G10 MAb and then Western blotted with anti-Stat3 MAb. (G) Anti-Stat3 (lanes 3, 4) and anti-Stat5 (lanes 1, 2) Western blotting of DNA affinity-purified complexes from preactivated PBL stimulated with IL-2 (lanes 1 and 3) or IL-6 (lanes 2 and 4).

3B; data not shown), confirming that these latter cytokines induce different complexes.

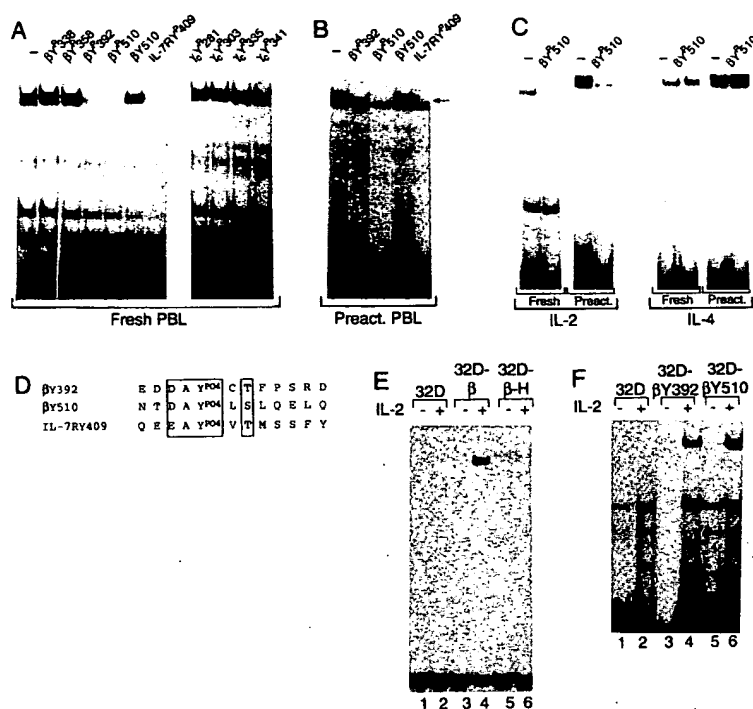
To characterize further the IL-2-induced complexes, we used DNA affinity purification of IL-2-induced complexes followed by Western blotting with antiphosphotyrosine antibodies (4G10) (Figure 3C, lanes 1–4; Figure 3D, lanes 1 and 2). The relative sizes of the bands identified in fresh and preactivated PBL (Figure 3, legend) corresponded to those seen by UV cross-linking (see Figure 2; Figure 2, legend). The greater apparent molecular weights observed in the UV cross-linking experiments likely result from the presence of both protein and the DNA probe in

the cross-linked adducts. Immunoblotting of the same affinity-purified material with an anti-Stat5 antiserum (Figure 3D, lanes 3 and 4) confirmed the presence of Stat5 in IL-2-induced complexes in fresh and preactivated PBL. The difference in molecular weights seen with fresh and preactivated PBL was striking. Although we do not fully understand the nature of the difference, it is possible that T cell activation induces posttranslational modifications of Stat5. Moreover, using a probe based on the cloned ovine Stat5 (Wakao et al., 1994), we have isolated three closely related human cDNAs, each of which encodes a somewhat different protein product. Both of the anti-Stat5 antibodies we have used recognize epitopes present in each of these forms of human Stat5, so it is not yet clear whether IL-2 activates one or all of the predicted protein products.

We observed that the complexes induced by IL-2 in fresh and preactivated PBLs have different reactivities to an antiserum, denoted Ab4, raised to the SH2 domain of Stat1 (Figure 3E). This antiserum is known to recognize only denatured forms of Stat1 and is not capable of supershifting authentic Stat1 in IFN-induced complexes (Rothman et al., 1994). Thus, its ability to supershift the IL-2/IL-7/IL-15 (Figure 3E; data not shown)-induced complexes in preactivated PBL is most likely based on cross-reactivity with another Stat protein. Since the anti-Stat5 antiserum recognized the complexes from both fresh and preactivated PBL (Figure 3A), while Ab4 recognized only the complex in preactivated PBL (Figure 3E), we investigated whether additional Stat proteins were activated only in preactivated PBL. Indeed, we found that IL-2 rapidly activated Stat3 in preactivated (Figure 3F, lanes 3 and 4) but not in fresh (lanes 1 and 2) PBL. Stat3 was previously shown to be activated in response to IL-6 (Akira et al., 1994) and epidermal growth factor (Zhong et al., 1994). By using DNA affinity purification and Western blotting with Stat5- and Stat3-specific antisera, we confirmed that Stat3 but not Stat5 was present in the IL-6-induced complex, whereas both Stat3 and Stat5 were in the IL-2-induced complex in preactivated PBL (Figure 3G). Thus, our data support the model that IL-2 activates Stat5 in fresh PBL, but both Stat3 and Stat5 in preactivated PBL. So far, we have found no evidence that Stat2, Stat4, or IL-4 Stat (Stat6) are components of the IL-2-induced complexes in either fresh or preactivated PBL.

#### Importance of Similar Motifs in IL-2R $\beta$ and IL-7R for Stat Protein Binding

The ability of Stat1 to bind to a motif spanning a phosphorylated tyrosine residue in the cytoplasmic domain of the IFN $\gamma$  receptor (Greenlund et al., 1994) prompted us to investigate whether the binding activity of IL-2-activated Stat proteins could be inhibited by peptides spanning phosphorylated tyrosines of IL-2R $\beta$  or  $\gamma_c$ . Of the 6 tyrosines in human IL-2R $\beta$ , 5 are conserved in mouse and rat. Only  $\beta$ Y338,  $\beta$ Y392, and  $\beta$ Y510 were phosphorylated in overexpression experiments in COS-7 cells (M. F. et al., unpublished data). Tyrosine-phosphorylated peptides spanning  $\beta$ Y392 or  $\beta$ Y510 inhibited nuclear factor binding in extracts from fresh PBL, with  $\beta$ Y510 being more effective (Figure 4A). In contrast,  $\beta$ Y338,  $\beta$ Y358 (spanning a tyrosine that



**Figure 4. The IL-2R $\beta$  and IL-7R Cytoplasmic Domains Share a Motif Capable of Binding the IL-2-Activated Stat Proteins**

Competition of binding of the IL-2-induced complexes in fresh (A) and preactivated (B) PBL by  $\beta$ Y392,  $\beta$ Y510, and IL-7RY409 tyrosine-phosphorylated peptides but not by unphosphorylated  $\beta$ Y510.

(A)  $\beta$ Y338,  $\beta$ Y358 and four  $\gamma_c$  phosphorylated peptides did not compete.

(B) Only the upper complex was competed (arrow).

(C) In fresh and preactivated PBL,  $\beta$ Y510 competed complexes induced by IL-2 (lanes 1-4) but not by IL-4 (lanes 5-8).

(D) Alignment of similar residues in the  $\beta$ Y392,  $\beta$ Y510, and IL-7RY409 peptides.

(E) IL-2 potentially induced nuclear complexes in 32D cells transfected with full-length IL-2R $\beta$  (Otani et al., 1992) (lane 4 versus 3) but not in cells transfected with the  $\beta$ -H mutant (lane 6 versus 5), which is truncated at amino acid 379 (Hatakeyama et al., 1989).

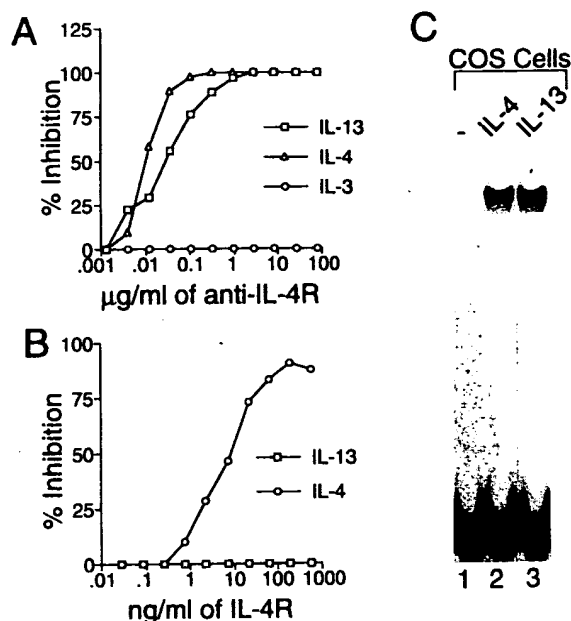
(F) Two IL-2R $\beta$  constructs in which all tyrosines except either Y392 (lanes 3 and 4) or Y510 (lanes 5 and 6) were mutated to phenylalanines could mediate Stat protein activation in 32D cells in response to IL-2 (lanes 4 and 6).

does not appear to be phosphorylated in cells), the phosphorylated peptides spanning each  $\gamma_c$  tyrosine, and a non-phosphorylated peptide spanning  $\beta$ Y510 had no effect (Figure 4A). Similar results were found using extracts from preactivated PBL, except that only the upper complex was eliminated when extracts were preincubated with  $\beta$ Y392 and  $\beta$ Y510 (Figure 4B). The peptide competitions were specific, since phosphorylated  $\beta$ Y510 did not inhibit formation of the nuclear complexes induced by IL-4 in fresh and preactivated PBL (Figure 4C).  $\beta$ Y392 and  $\beta$ Y510 exhibited sequence similarities (Figure 4D). Since IL-7 and IL-2 appeared to activate the same Stat proteins, we examined the sequence of the cloned IL-7 binding protein (IL-7R) for a similar motif and found one spanning Y409 (Figure 4D). Importantly, a tyrosine-phosphorylated peptide spanning IL-7RY409 also inhibited Stat protein binding (Figures 4A and 4B).

Interestingly, the IFN $\gamma$ R sequence (TSFGYPO4DKPH; Greenlund et al., 1994) that binds Stat1 and the IL-4R sequences (GEXGYPO4KXFXXXL; Hou et al., 1994) that bind IL-4 Stat both contain a lysine C-terminal to the phosphorylated tyrosine; however, no positively charged residue was present in the IL-2R $\beta$ /IL-7R motifs (Figure 4D). It will be interesting to determine in detail the sequence requirement for receptor-Stat protein association. The importance of the IL-2R $\beta$  motifs was indicated by the dramatic decrease in IL-2-mediated activation of Stat proteins in 32D cells transfected with a truncated IL-2R $\beta$  construct in which Y392 and Y510 were deleted as compared with cells transfected with wild-type IL-2R $\beta$  (Figure 4E). Moreover, constructs in which all tyrosines except either Y392 or Y510 were mutated could mediate Stat protein activation in response to IL-2 (Figure 4F), indicating that either Y392 or Y510 alone was sufficient for this effect.

#### Role of IL-4R in IL-4- and IL-13-Mediated Signaling: Activation of Stat Proteins by IL-4 and IL-13 in Cells Lacking $\gamma_c$ and Jak3

Finally, we sought to clarify the basis for activation of identical Stat proteins by IL-4 and IL-13. Based on the ability of a nonfunctional IL-4 mutant (Y124D) to block both IL-4 (Kruse et al., 1992) and IL-13 (Zurawski et al., 1993) action, it has been concluded that the IL-4 and IL-13 receptors share a common component (Zurawski et al., 1993). Although  $\gamma_c$  was hypothesized to be this shared component (Kondo et al., 1993; Russell et al., 1993), we have not been able to confirm this hypothesis (data not shown). Instead, our data suggest that the known 140 kDa IL-4 binding protein (IL-4R) itself is the shared component. Of 10 anti-IL-4R antibodies tested, only those that blocked IL-4 action also blocked IL-13 action. One of these is shown in Figure 5A (see also Figure 5A legend). None of the antibodies inhibited IL-3-induced proliferation of TF-1 cells (Figure 5A) nor IL-2/IL-7 actions on IL-2/IL-7-responsive cells (data not shown). In contrast with the inhibition of IL-13 action by anti-IL-4R antibodies, soluble IL-4R did not inhibit IL-13 action (Figure 5B). This suggests that the IL-4R is not the primary binding protein for IL-13. However, the fact that both IL-4 Y124D (Zurawski et al., 1993) and antibodies to IL-4R (Figure 5A) inhibit IL-13 action suggests that the IL-4R is the shared component of the IL-4 and IL-13 receptors. Furthermore, IL-4 and IL-13 induced similar nuclear complexes in COS-7 cells (Figure 5C) which express IL-4R but not  $\gamma_c$  (Russell et al., 1993; data not shown). Additionally, even though normal human monocytes express  $\gamma_c$  (Bosco et al., 1994; Epling-Burnette et al., 1995), two monocytic cell lines (THP-1 and Mono Mac 6) lack  $\gamma_c$  expression but nevertheless can respond to IL-4 (Takeshita et al., 1992; de Wit et al., 1994; Kotanides and Reich,



**Figure 5.** Blocking of IL-13 Action by a MAb to the IL-4R, and Induction of Similar Nuclear Complexes by IL-4 and IL-13 in Cells Lacking  $\gamma_c$ . (A) A MAb to the IL-4R (MAb 25452.11; R and D Systems, produced using purified recombinant soluble IL-4R as an immunogen) inhibited IL-4 (0.2 ng/ml)- and IL-13 (6 ng/ml)- but not IL-3 (0.3 ng/ml)-induced proliferation of TF-1 cells. Of 10 MAbs tested, all had concordant effects on IL-4 and IL-13 (i.e., IL-13 action was inhibited if and only if IL-4 action was inhibited). One polyclonal antibody and four MAbs potently inhibited, four MAbs could inhibit but required approximately 10,000 higher concentrations, and one MAb did not inhibit at any concentration tested. (B) Soluble IL-4R (0–1000 ng/ml) inhibited IL-4 but not IL-13 action. (C) IL-4 (lane 2) and IL-13 (lane 3) induced complexes of identical mobility in COS-7 cells. In (A) and (B), proliferation assays were performed with TF-1 cells ( $1 \times 10^4$  cells in 100  $\mu\text{l}$  in each well). Antibodies or soluble IL-4R were added simultaneously to the cytokines. After a 2 day incubation, cells were pulsed with 0.25  $\mu\text{Ci}$ /well of [ $^3\text{H}$ ]thymidine for 2 hr and the incorporated cpm determined. Results were similar if TF-1 cells were preincubated with IL-4R MAb or soluble IL-4R prior to addition of cytokine. The ED<sub>50</sub>s for IL-3, IL-4, and IL-13 are 0.15 ng/ml, 0.1 ng/ml and 3 ng/ml, respectively.

1993). Thus, consistent with an earlier hypothesis that two types of IL-4 receptors may exist (Rigley et al., 1991), functional IL-4 receptors contain IL-4R plus either  $\gamma_c$  (Kondo et al., 1993; Russell et al., 1993) or a second protein that we shall denote  $\gamma'$  and which we hypothesize may be the primary IL-13 binding protein. The existence of  $\gamma'$  is additionally suggested by the ability of  $^{125}\text{I}$ -IL-4 to affinity-label a species smaller in molecular weight than the IL-4R in COS cells (Galizzi et al., 1990). Moreover, the stoichiometry of IL-4 binding to IL-4R is 1:1 (Hoffman et al., 1994), suggesting the requirement for a second receptor chain to fit with the general model that four helix bundle cytokines bind to receptors comprising homodimers or heterodimers rather than monomeric receptors (Leonard, 1994; Stahl and Yancopoulos, 1993; Kishimoto et al., 1994). Although both types of IL-4 receptors mediate Stat protein activation, it is possible that they differ in some of the signals transduced; if so, this would represent a mechanism by

which IL-4 can induce different signals (cytokine pleiotropy) according to the type of receptor expressed.

## Discussion

The growth, differentiation, and functional activities of lymphocytes are regulated by the interaction of specific cytokines with their cell surface receptors. A variety of cytokines exhibit actions as T cell growth factors; we have studied four of these cytokines, IL-2, IL-4, IL-7, and IL-15, all of whose receptors on T cells contain the common  $\gamma$  chain. In addition, we have studied IL-13, a cytokine whose receptor does not contain  $\gamma_c$ . IL-13 does not stimulate T cells, yet it shares actions with IL-4 on NK cells and B cells. By investigating the Stat proteins activated by these cytokines, we have gained insights into some of the mechanisms underlying their shared and distinct actions.

Previously, IL-2 was shown to induce a nuclear complex in a rodent T cell line (Gilmour and Reich, 1994). Interestingly, the complex exhibited similar mobility with that induced by prolactin in the same cell line (Gilmour and Reich, 1994). However, it was not determined whether prolactin (which has been shown to activate Stat5; Wakao et al., 1994) and IL-2 activated the identical Stat proteins. Beadling et al. (1994) detected an IL-2-activated Stat protein in human PBL but did not elucidate its identity. In investigating the identity of Stat proteins within IL-2-induced complexes, we found that IL-2 can activate different combinations of Stat proteins in normal cells under different physiological conditions. This has not been reported for any other cytokine. Based on the difference in UV cross-linked adducts in fresh versus preactivated PBL, it was initially surprising that the complexes from both fresh and preactivated PBL contained Stat5. PHA-mediated posttranslational modifications presumably contribute to the size differences seen; moreover, the different complexes at least partially resulted from the involvement of Stat3 in the preactivated complex.

It remains to be determined whether the complexes formed in fresh and preactivated PBL have different functions: for example, it is conceivable that complexes containing only Stat5, as are found in freshly isolated cells, play a role in augmenting cytolytic activity of NK cells and in differentiation of T cells. In contrast, the broader range of potential complexes in IL-2-stimulated preactivated PBL (i.e., Stat5 and Stat3 homodimers and Stat3–Stat5 heterodimers), could potentially mediate the triggering of additional signals in IL-2-stimulated preactivated PBL, including, for example, its major action in promoting T cell proliferation. The induction of distinct complexes in different cell populations may be one mechanism by which cytokines exert pleiotropic actions. The existence of alternative forms of Stat5 may additionally contribute to the complexity of IL-2 signals.

The complexes induced by IL-2 were the same as those induced by IL-7 and IL-15, whereas those induced by IL-4 were the same as those induced by IL-13. These observations help to explain the basis of cytokine redundancy and led us to elucidate how individual Stat proteins can be activated by more than one cytokine (Figure 6). Interest-

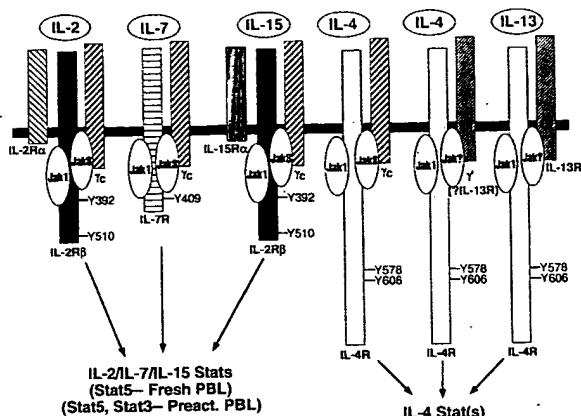


Figure 6. Schematic Model Showing that IL-2, IL-7, and IL-15 Activate Identical Stat Proteins Due to the Existence of Similar Motifs in IL-2R $\beta$  and IL-7R

IL-4 activates Stat proteins via IL-4 receptors containing IL-4R plus  $\gamma_c$  or IL-4R plus  $\gamma'$  (we speculate that  $\gamma'$  is the IL-13R); IL-13 activates IL-4 Stat through receptors that appear to contain IL-13R and IL-4R. IL-2R $\beta$  Y392 and Y510 and IL-7R Y409 were identified in this study, whereas IL-4R Y578 and Y606 were identified by Hou et al. (1994).

ingly, although the receptors for IL-2, IL-4, and IL-7 share  $\gamma_c$  and each of these cytokines activate Jak1 and Jak3, IL-2 and IL-7 activate different Stat proteins than does IL-4. It therefore appears that the primary role of  $\gamma_c$  is to recruit Jak3 (Boussiotis et al., 1994; Russell et al., 1994; Miyazaki et al., 1994), while IL-2R $\beta$ , IL-7R, and IL-4R determine which Stat protein(s) will be activated. It has been reported recently that signaling via receptors that contain  $\gamma_c$  can prevent the induction of anergy in T cells (Boussiotis et al., 1994). It will be interesting to determine the role that Stat proteins play in this process. In COS-7 cells, which do not express  $\gamma_c$  or Jak3 (Russell et al., 1993; data not shown), the ability of an alternative functional form of the IL-4 receptor (Figure 6) to mediate Stat protein activation represents an additional mechanism for regulating cytokine-specific signals.

Thus, we have found that Stat3 and Stat5 can be activated by IL-2, IL-7, and IL-15. In addition, we have identified two mechanisms by which different cytokines can activate the same Stat proteins, either the sharing by receptors of a common chain (the IL-2 and IL-15 receptors share IL-2R $\beta$  and the IL-4 and IL-13 receptors share IL-4R) or by distinct receptor chains that share a common motif (IL-2R $\beta$  and IL-7R). The activation of the same Stat proteins by distinct cytokines presumably contributes to the generation of redundant cytokine actions. In contrast, the activation of nonidentical sets of Stat proteins by a single cytokine (e.g., the activation of Stat5 in fresh PBL but both Stat3 and Stat5 in preactivated PBL) presumably contributes to cytokine pleiotropy. Importantly, however, IL-4 can act as a T cell growth factor even though it induces a different complex than do IL-2, IL-7, and IL-15. It is, therefore, possible that in vivo more than one type (but presumably not every type) of Stat protein complex can serve to activate genes essential to promote T cell proliferation.

Alternatively, there may be more than one molecular mechanism by which T cell growth can be promoted.

#### Experimental Procedures

**Cells, Cell Culture, Preparation of Nuclear Extracts, and EMSAs**  
PBLs were isolated from normal donors by standard methods, immediately stimulated with cytokines, and nuclear extracts prepared; alternatively, cells were preactivated by culturing for 72 hr with PHA (1  $\mu$ g/ml, Boehringer-Mannheim) and rested overnight in medium containing 10% fetal calf serum without IL-2, prior to stimulation with cytokines. YT cells were cultured in RPMI 1640 containing 10% fetal calf serum. CTLL-2 were cultured in a similar fashion, except that culture was supplemented with  $5 \times 10^{-5}$  M 2-mercaptoethanol and 20 U/ml IL-2. For CTLL-2, cells were grown in the absence of IL-2 for 4 hr prior to stimulation with IL-2. Purified NK cells were prepared as described by Yamauchi and Bloom (1993). Purified T cells (ranging from 92%–96% pure) were isolated by antibody-mediated depletion on magnetic beads. Nuclear extracts were prepared as described (Lin et al., 1993) and 4 or 10  $\mu$ g were used in EMSAs. EMSAs were performed as previously described (Lin et al., 1993).

#### Antisera

An anti-human Stat5 antiserum (R1146) was produced by immunizing rabbits with a peptide corresponding to amino acids 66–80 of human Stat5 (NH<sub>2</sub>-CQELQKKAHQVGEDG-COOH; the Cys at the N terminus was added), synthesized (by G. Poy, National Institutes of Health) on an Applied Biosystems 431A peptide synthesizer using a MAP 8 branched resin (Applied Biosystems). R1146 was used for anti-Stat5 supershift and immunoprecipitation experiments. Monoclonal antibodies (MAbs) to Stat2, Stat3, Stat4, and Stat5 were from Transduction Laboratories (Lexington, Kentucky). Antisera for p84/p91 (Stat1) and p91 (Stat1a) were from Santa Cruz Biotechnology. Monoclonal anti-phosphotyrosine antibody 4G10 was from Upstate Biotechnology.

#### UV Cross-Linking

For UV cross-linking experiments, 30–40  $\mu$ g of nuclear extracts were preincubated with 4  $\mu$ g of poly dI:dC in binding buffer prior to addition of 300,000 to 400,000 cpm (1 ng) of probe (the bottom strand of the IL-2R $\beta$  oligonucleotide, 5'-CATTTCATGAATTCGGATCC-3' [this is complementary to the strand shown in the Figure 1D legend], was labeled by specific priming in the presence of bromodeoxyuridine and [<sup>32</sup>P]dATP). Samples were irradiated for 30 min at 70 W in a Hoefer UV cross-linker and resolved on 7.5% SDS gels. For cold competition, the unlabeled oligonucleotide was preincubated for 20 min with nuclear extracts prior to addition of probe.

#### Affinity Purification and Western Blotting

Nuclear extracts (200  $\mu$ g for IL-2-stimulated preactivated PBL, 400  $\mu$ g for IL-6-stimulated preactivated PBL, and 800  $\mu$ g for IL-2-stimulated fresh PBL) were incubated on ice for 15 min in a final volume of 200  $\mu$ l binding buffer (12 mM HEPES [pH 7.9], 20 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 1 mM AEBSF, 10% glycerol) containing 4–16  $\mu$ g of poly dI:dC (Boehringer-Mannheim). Then 40–160 ng of biotinylated double-stranded oligonucleotides containing the GAS motifs from IL-2R $\beta$  (5'-biotin-GATCGAATTCGAAATGGATCGAATTCATGGAATGGATCGAATTCATGGAATGC-3') or Fc $\gamma$ RI (5'-biotin-GATCGATTTCCAGAAAAGGATCGATTTCCAGAAAAGGATCGTATTTC CAGAAAATGC-3') genes (Midland Reagent Company; the three copies of the GAS motif in each oligonucleotide are underlined) was added and the incubation continued for 15 min at room temperature. The reaction was added to streptavidin magnetic beads (Promega) that had been washed three times in washing buffer (12 mM HEPES [pH 7.9], 100 mM KCl, 1 mM EDTA, 0.5 mM DTT, 12% glycerol, 1 mM AEBSF, 0.05% NP40, 100 mg/ml bovine serum albumin) and incubated for 30 min on ice. The beads were then washed three times with washing buffer containing 33  $\mu$ g/ml poly dI:dC and the bound proteins eluted with Laemmli sample buffer, separated on 8% SDS-PAGE (Novex), blotted to Immobilon-P membranes, and Western analyses performed using either 4G10, or with anti-Stat3 or anti-Stat5 MAbs, and developed using enhanced chemiluminescence (Amersham).



# Immunoprecipitation and Western Blotting with Anti-Phosphotyrosine Antibodies

Cells ( $5-10 \times 10^6$ ) were stimulated or not stimulated with IL-2 (2 nM), washed twice with phosphate-buffered saline, and lysed with 10 mM Tris (pH 7.5) containing 2 mM EDTA, 0.15 M NaCl, 0.875% Brij 96, 0.125% Nonidet P40, 0.4 mM NaVO<sub>3</sub>, 1 mM AEBSF, 2.5 mM leupeptin, 2.5 mM aprotinin. Immunoprecipitations were performed using 4G10 MAb and protein A-Sepharose beads (Pharmacia) at 4°C for 4 hr. Samples were washed four times with lysis buffer, and boiled in 2x Laemmli sample buffer. Proteins were analyzed on 8% SDS-PAGE, transferred to Immobilon-P membranes, blotted with anti-Stat3 antibody or 4G10, and developed with enhanced chemiluminescence.

## Peptide Competition Experiments

Phosphorylated and nonphosphorylated peptides for competition experiments were synthesized using an Applied Biosystems 431A peptide synthesizer using Fmoc chemistry and HBTU activation. Fmoc phosphotyrosine was from Nova Biochemical. Peptides were purified by reverse phase C18 high pressure liquid chromatography and quantitated using a PicoTag system (Waters). For the peptide inhibition experiments, 100  $\mu$ M of phosphorylated or nonphosphorylated peptides were preincubated with nuclear extracts for 30 min at room temperature prior to addition of probe. For  $\beta$ Y510 and IL-7RY409, as little as 20  $\mu$ M tyrosine-phosphorylated peptide inhibited binding, whereas  $\beta$ Y392 required 100  $\mu$ M to see complete inhibition. Correctness of peptides was confirmed by mass spectroscopy and concentrations determined by quantitation with the PicoTag system.

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#### Note Added in Proof

Support for the models of IL-4 and IL-3 receptors shown in Figure 6 is also provided by the following recent publications:

- Obiri, N. I., Debinski, W., Leonard, W. J., and Puri, R. K. (1995). Receptor for interleukin 13: interaction with interleukin 4 by a mechanism that does not involve the common chain shared by the receptors for interleukins 2, 4, 7, 9, and 15. *J. Biol. Chem.* 270, 8797-8804.
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- Vita, N., Lefort, S., Laurent, P., Caput, D., and Ferrara, P. (1995). Characterization and comparison of the interleukin 13 receptor with the interleukin 4 receptor on several cell types. *J. Biol. Chem.* 270, 3512-3517.